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Role of Disulfide Bonds in Maintaining the Structural Integrity of the Sheath of *Leptothrix discophora* SP-6

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Isolated sheaths of *Leptothrix discophora* SP-6 (ATCC 51168) were tested for susceptibility to degradation by a variety of chemical denaturants and lytic enzymes and found to be resistant to many reagents and enzyme treatments. However, disulfide bond-reducing agents such as dithiothreitol (DTT), β-mercaptoethanol, sodium cyanide, and sodium sulfite degraded the sheath, especially at elevated pH (pH 9) and temperature (50°C). DTT and β-mercaptoethanol caused more rapid degradation of the sheath than cyanide or sulfite. Treatment of the sheath with 1 N NaOH resulted in rapid breakdown, while treatment with 1 N HCl resulted in slow but significant hydrolysis. Transmission electron microscopy showed that the 6.5-nm fibrils previously shown to be an integral structural element of the sheath fabric (D. Emerson and W. C. Ghiorse, J. Bacteriol. 175:7808–7818, 1993) were progressively dissociated into random masses during DTT-induced degradation. Quantitation of disulfide bonds with DTT showed that the sheaths contained approximately 2.2 μmol of disulfides per mg of sheath protein. Reaction with 5,5′-dithio-*bis*-(2-nitrobenzoic acid) showed that sheaths also contained approximately 0.8 μmol of free sulfhydryls per mg of protein. A sulfhydryl-specific fluorescent probe (fluorescein 5-maleimide) showed that the free sulfhydryls in sheathed cell filaments were evenly distributed throughout the sheath. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis autoradiography of [^{14}C]iodoacetamide-labeled sheaths and DTT-dissociated sheath fibril suspensions showed that the majority of ^{14}C-labeled sulfhydryls in the sheaths did not enter the gel. However, low-molecular-mass silver-staining bands (14 to 45 kDa) did appear in the gels after iodoacetic acid or iodoacetamide alkylation of the dissociated fibrils. These bands did not stain with Coomassie blue. Their migration in gels was slightly affected by digestion with pronase. The fibrils contained 20 to 25% protein. These results confirm that the sheath fibrils consist of high-molecular-weight heteropolysaccharide-protein complexes. We hypothesize that proteins in the fibril complexes provide interfibril cross-linking to maintain the structural integrity of the sheath.

Several previous studies of eubacterial sheaths have focused on their ultrastructure and gross chemical composition (1, 20, 31, 33, 34, 42). These tube-like extracellular structures are composed principally of heteropolysaccharides in a fabric of fine fibrils, which appear to be the primary structural elements (11, 20, 31). Bacterial sheaths are very durable structures. Indeed, fossilized eubacterial sheaths are frequently reported in the geologic record (17). It seems likely that covalent cross-linking could play a major role in maintaining the structure. However, the manner by which the macromolecular components of the sheath fabric are cross-linked has not been elucidated.

In an accompanying paper (15), we show that the fine structure and gross chemical composition of the sheath of *Leptothrix discophora* SP-6 closely resemble those of its close relative, *Sphaerotilus natans*. The sheath fabric is composed of 6.5-nm fibrils containing heteropolysaccharide and protein. In addition, we found that the sheath of *L. discophora* SP-6 contains a relatively high concentration of cysteine residues (6 mol%), suggesting that disulfide bonds could play an important role in maintaining the structure of the sheath fabric.

Here we show that the structural integrity of the *L. discophora* SP-6 sheath was highly susceptible to disintegration by disulfide bond-reducing agents. While a wide variety of lytic enzymes and chemical reagents failed to degrade its structure, the disulfide-reducing agents rapidly disintegrated the sheath into a suspension of dissociated structural fibrils. We hypothesize that disulfide bonds covalently cross-link fibrils together, thus maintaining the integrity of the sheath fabric. (A preliminary report of this work was presented previously [13].)

MATERIALS AND METHODS

Bacterial culture and sheath isolation. The isolation and growth of *L. discophora* SP-6 (ATCC 51168) on a mineral salts-vitamins-pyruvate medium under conditions that promote maintenance of sheath formation have been described previously (14). In the present studies, SP-6 cultures were grown in batches of 2 to 2.5 liters in 5-liter carboys aerated by bubbling with air as described previously (15). Cell-free sheaths were prepared with either sodium dodecyl sulfate (SDS) or *N*-lauroylsarcosine (Sarkosyl) as specified by the sheath isolation procedure described previously (15).

Sheath dissociation assay and treatments with disulfide and reducing reagents. An optical density assay modeled after that described by Beveridge et al. (5) was used to follow the degradation of the sheath after treatment with the disulfide bond-reducing reagents dithiothreitol (DTT), β-mercaptoethanol, sodium cyanide, and sodium sulfite and other reagents as described below. In the disulfide bond-reducing reagent treatments, 0.4 ml of isolated sheath suspension in distilled water, containing approximately 0.35 mg (dry weight) of isolated sheaths, was placed in a 2.0-ml cuvette containing 0.5 ml of reaction buffer (either 40 mM HEPES [α1-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], pH 7, or 40 mM bicine buffer, pH 9). Enough deionized water was added to make the total volume 1.0 ml. The reaction was started by adding a

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prescribed volume of a stock solution of the disulfide-reducing reagent (<0.1 ml). A zero-time reading of optical density at 600 nm (OD_{600}) was made in a Beckman model 25 spectrophotometer against a cuvette lacking reagent. The experimental cuvette was incubated for a period of 30 to 40 min in a water bath at the desired temperature, usually 40 or 50°C; and periodically the cuvette was removed from the water bath, the contents were mixed thoroughly with a Pasteur pipet, and the OD_{600} of the mixed suspension was recorded.

In one experiment, the release of protein during sheath degradation by various concentrations of DTT was estimated by measuring the amount of protein released into the supernatant following centrifugation at 8,800 × *g* for 5 min. This was done by treating 1.0 ml of sheath suspension containing approximately 0.8 mg (dry weight) of isolated sheaths (0.2 mg of protein) with 0, 0.5, and 5 mM DTT in 20 mM bicine buffer, pH 9.0, for 30 min at 40°C. The change in OD_{600} was followed for 30 min. The sample was then centrifuged for 5 min at 8,800 × *g* in an Eppendorf microcentrifuge, and the supernatant was removed and assayed for protein by the Bio-Rad-Bradford assay as described previously (15).

Treatments with other reagents. The ability of a variety of enzymes, solvents, and other denaturants to degrade the sheath structure was determined by measuring the change in OD_{600} by the sheath dissociation assay described above and by observing changes in phase density of the sheath by phase contrast light microscopy as described previously (15). In most experiments, 1 ml of sheath suspension in deionized water or buffer containing approximately 0.35 mg (dry weight) of isolated sheaths was reacted with 1 ml of the given reagent under prescribed conditions. All enzymes were purchased from Sigma Chemical Co. (St. Louis, Mo.) except trypsin, which was from Millipore Corp. (Bedford, Mass.). Pronase (100 to 150 μg/ml) from *Streptomyces griseus* type XIV was used as described by Narahashi (27). Papain (100 μg/ml), from papaya latex, was dissolved in 0.05 M Tris buffer, pH 8.0, and activated by 0.05 M cysteine in 0.02 M EDTA adjusted with NaOH to pH 8.0. Subtilisin (1 mg/ml) from *Bacillus subtilis* was dissolved in 0.025 M bicine buffer, pH 9.0. Trypsin (1 mg/ml) was dissolved in 0.001 M HCl and reacted with sheath in 0.04 M Tris buffer, pH 8.1, with 0.01 M $CaCl_2$ added. Casein was used as a positive control to ensure that the proteases were active under the conditions of the experiment (32). Hyaluronidase from bovine testes was applied, and hyaluronic acid was assayed according to the recommendations of Matthews (24) with hyaluronic acid (Sigma) as a standard. Chondroitinase ABC from *Proteus vulgaris* was applied, and chondroitin sulfate was assayed (43) with chondroitin sulfate as standard. *N*-acetylhexosaminidase from jack beans was employed according to the method of Srivastava et al. (38). *N*-acetylhexosamines were assayed as previously described (15).

To determine whether oxygen radicals could depolymerize the sheath, a superoxide-generating system using hypoxanthine or xanthine oxidase was used according to procedures described by McCord (25).

Isolated sheath suspensions in deionized water also were treated with either 45% phenol (30), 5% trichloroacetic acid (33), or a chloroform-methanol-water mixture (1:2:0.8) (6). After treatment with these reagents, a sample of the mixture was examined by phase contrast microscopy. The mixture was then centrifuged at 8,800 × *g* for 4 min, and the supernatants were analyzed for protein, reducing sugar, or lipid as described previously (15).

Other potentially disruptive reagents were tested by the sheath dissociation assay described above. These included 6 M urea, 3 M guanidine thiocyanate, 6 M guanidine-HCl, 2 M NaCl, 50 mM EDTA, and 0.3 M $NaBH_4$ in 20 mM bicine buffer, pH 9.0, under an N_2 atmosphere. Isolated sheath suspensions in deionized water were mixed 1:1 with the reagent at twice the final concentration and then incubated in a water bath at 40 or 50°C. OD_{600} was monitored for prescribed times ranging between 1 and 12 h.

TEM. Isolated sheath suspensions were examined by the metal-shadowing transmission electron microscopy (TEM) technique after treatment with 0, 0.5, 1.0, 2.0, and 5.0 mM DTT at pH 9 and 40°C. The treated samples were mixed thoroughly, and a drop of the suspension was removed. A Formvar-coated copper EM grid was floated on the drop for 1 min. The grid was then removed, blotted, and air dried. Grids were shadowed with Pt-Pd (80:20) in a Balzers BAE 080 vacuum evaporator and observed with a Philips 300 TEM operating at 80 kV as described previously (15).

Determination of SH and disulfide content. Quantitative assays for free sulfhydryl (SH) groups were done with 5,5′-dithio-*bis*-(2-nitrobenzoic acid) (DTNB) (22). The approximate concentration of disulfide bonds in isolated sheaths was determined by measuring the increase in UV A_{310} (21) of a DTT solution after reaction with the sheath suspension. In this procedure, samples of sheath suspension (containing from 0.1 to 0.2 mg of protein) were treated with 1 mM DTT in 2.0 ml of a buffer consisting of 20 mM bicine, pH 9.0, plus 1 mM EDTA in a test tube (1 by 10 cm). The assay was started by adding the DTT and mixing the contents of the test tube. The mixture was incubated in a water bath at 40°C for 15 min and then filtered through a 0.22-μm-pore-size filter; filtration was necessary because of light scattering by the intact sheaths and dissociated sheath fibrils in the suspension. The change in A_{310} of the filtrate was measured in the spectrophotometer. Blanks containing all the assay components except the sheath suspension were also monitored to correct for autooxidation of the DTT. Solutions with known amounts of oxidized glutathione were reacted with DTT as a positive control in the assay procedure. The quantity of disulfides was estimated by using a molar extinction coefficient for DTT of 110 at 310 nm (21).

In some cases, free SH groups in the sheath suspensions were alkylated by iodoacetic acid, which carboxymethylates free thiol groups of proteins (10). In the alkylation procedure, 0.3 ml of 1.5 M Tris buffer, pH 8.8, and 0.1 ml of 1.0 M iodoacetic acid (Aldrich Chemical Co., Milwaukee, Wis.) were mixed with 10 ml of sheath suspension in deionized water containing approximately 8 mg (dry weight) of sheath material. The mixture was gassed with N_2 for 2 to 3 min and then incubated in the dark for 30 min at room temperature. The alkylating reagents were removed by dialysis (6,000- to 8,000-molecular-weight cutoff) overnight against deionized water or by washing the sheaths in deionized water four times by centrifugation (8,800 × *g*, 5 min, room temperature).

FM reagent. A fluorescent probe reagent for detecting free SH groups in the sheath was prepared by using a concentration of 20 μM fluorescein 5-maleimide (FM) (Molecular Probes, Eugene, Oreg.) in 50 mM sodium phosphate buffer, pH 7.0. By varying the FM concentration, the optimum concentration (20 μM) for fluorescence brightness was determined to be a 25 M excess of FM relative to the quantity of SH in the sheath, as estimated by the DTNB assay (0.8 μM). For staining sheaths, a deionized water-washed suspension containing sheathed *L. discophora* SP-6 filaments was centrifuged and resuspended in the FM reagent for 10 min in the dark at room temperature. To verify the specificity of the FM reagent for SH groups, sheaths were alkylated with iodoacetic acid and then treated with the FM reagent as described above. FM-treated samples were viewed in a Zeiss standard microscope under phase

contrast and epifluorescence optics with the appropriate filter combination (450- to 490-nm excitation filter, 520-nm longpass emission filter) for fluorescein.

PAGE. To determine what effect dissociation with DTT had on releasing proteins associated with the sheath, 1.0 to 1.5 ml of sheath suspension containing 250 μg of protein was centrifuged in the Eppendorf microcentrifuge (4 min, 8,800 × *g*) and resuspended to the original volume in a reaction mixture containing 20 mM bicine buffer, pH 9.0, and 10 mM DTT. The mixture was incubated for 30 min at 40°C to complete the dissociation reaction and then centrifuged for 15 min to remove nondissociated material. A portion of the dissociated material was alkylated with iodoacetic acid as described above. In some cases 1.0 M iodoacetamide (Sigma) was substituted for 1.0 M iodoacetic acid, but all other conditions remained the same. The alkylated material was dialyzed overnight against deionized water and concentrated under N_2 gas at 40°C, as described above, prior to being mixed with SDS-polyacrylamide gel electrophoresis (PAGE) treatment buffer. The discontinuous buffer system of Laemmli (23) was used with a 12.5% polyacrylamide resolving gel and a 4% stacking gel. The treatment buffer contained 60 mM bicine buffer, pH 8.8, 30% glycerol, 6% SDS, 40 mM DTT, and 0.02% bromophenol blue. Sheath samples were mixed 2:1 with treatment buffer and heated at 100°C for 75 s prior to being loaded on the gel. After electrophoresis, gels were stained with a silver-staining kit according to the manufacturer's instructions (Bio-Rad, Redwood, Calif.).

PAGE gels also were stained for glycoprotein by a periodic acid-Schiff (PAS) staining procedure (35) employing commercially available Schiff reagent (Sigma), and with horseradish peroxidase (Sigma) as a positive control.

Dissociated and alkylated sheaths and fibrils were treated with pronase prior to SDS-PAGE by mixing 1 ml of a DTT-dissociated, alkylated, and dialyzed sheath mixture with 100 μl of a reaction buffer containing 5 mM phosphate buffer, pH 7.5, and 1 mM $CaCl_2$. Fifty micrograms of pronase was added, and the mixture was incubated at 37°C for 5 h. The sample was dialyzed overnight against deionized water at 5°C and concentrated to a volume of 0.1 ml under a stream of N_2 gas. The samples were suspended in treatment buffer and subjected to SDS-PAGE as described above.

Autoradiography. To label SH groups for autoradiography of SDS-PAGE-separated material on gels, whole sheaths and dissociated sheath fibrils were labeled by covalently modifying them with [^{14}C]iodoacetamide (Amersham) according to procedures described by Hackstedt et al. (19) and Couche et al. (9). All concentrations given below are final concentrations unless otherwise stated. Whole sheaths were labeled by placing a sheath suspension (approximately 0.85 mg [dry weight]) in 40 mM Tris buffer, pH 8.0, adding 2.5 μCi of [^{14}C]iodoacetamide, and incubating the mixture for 1 h at 4°C. The labeling reaction was quenched by addition of 25 mM unlabeled iodoacetamide and further incubation at 4°C for 1.5 h. The ^{14}C-labeled sheaths were washed four times by centrifugation in deionized water, and after the final wash, the pellet was mixed with the SDS-PAGE treatment buffer and subjected to electrophoresis as described above.

Sheath fibrils were labeled by centrifuging a suspension containing 0.85 mg (dry weight) of whole sheaths and resuspending the pellet in 1.0 to 1.5 ml of 30 mM bicine buffer, pH 8.8. The sheaths were centrifuged again, and the pellet (approximately 0.4 ml) was treated by addition of 100 μl of 20 mM DTT and incubation in a water bath at 50°C for 15 min to dissociate the sheath fibrils. The dissociated fibrils were then labeled by adding 2.5 μCi of [^{14}C]iodoacetamide and incubating the mixture at 4°C for 1 h. The labeled material was then subjected to SDS-PAGE as described above. As a negative control, a suspension of sheaths (0.90 mg [dry weight]) was dissociated and alkylated with [^{12}C]iodoacetamide as described above. The ^{12}C-alkylated material was treated with 2.5 μCi of [^{14}C]iodoacetamide and incubated at 4°C for 1 h. The samples were then subjected to electrophoresis as described above. Duplicate gels were run for each set of samples; one gel was stained with the silver-staining kit, and the other was dried on a vacuum gel dryer (Hoefer Instruments, San Francisco, Calif.) and placed on a sheet of Kodak X-Omat film for autoradiography. The autoradiogram was exposed for 15 days at 4°C in the dark before development by standard procedures.

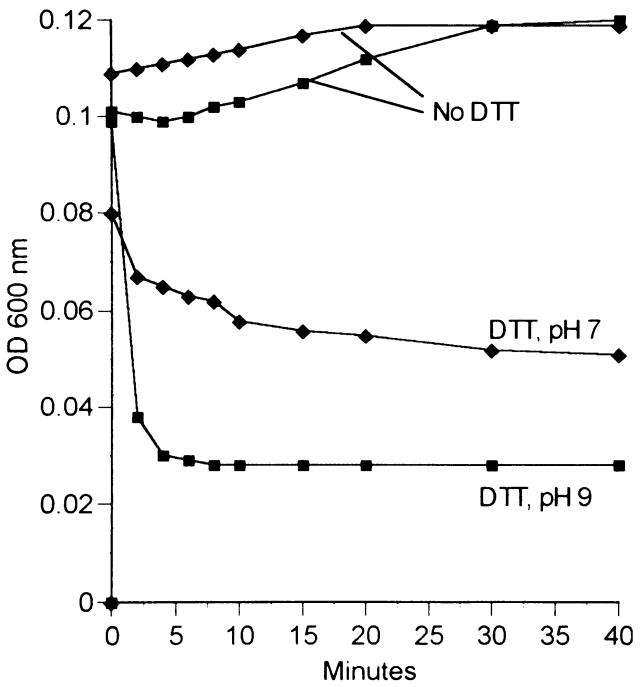

FIG. 1. Effect of 5 mM DTT on sheaths isolated from *L. discophora* SP-6 at pH 7.0 and pH 9.0, 50°C, as determined by decrease in OD_{600}.

RESULTS

Effect of disulfide bond-reducing agents. Treatment of the sheath suspensions with 5 mM DTT at pH 7 and 50°C caused an approximately 25% decrease in OD_{600} (Fig. 1). After centrifugation, the DTT-treated sheaths appeared intact but were less phase dense in the phase contrast microscope than the untreated controls. Increasing the pH to 9.0 in the presence of 5 mM DTT resulted in an approximately 70% decrease in OD_{600} within 5 min. Only small fragments of intact sheaths were then visible in the microscope. This pH effect is to be expected because DTT is known to be a more effective reducing agent at alkaline pH (22).

Treatment with hot trichloroacetic acid caused no apparent structural changes in the microscopic appearance of isolated sheaths, and no reducing sugar was detectable in the trichloroacetic acid extract. Hot-phenol extraction caused the sheaths to form a condensed layer at the phenol-water interface; however, there was no apparent change in phase density of the sheaths observed in the light microscope. Chloroform-methanol extraction did extract some lipid material from the sheaths,

TABLE 1. Agents that caused little or no change in light-microscopic phase density or decrease in light OD_{600} in the sheath of *L. discophora* SP-6[a]

Agent (concn)	Agent (concn)
Enzymes	Detergents
Lysozyme (150 μg/ml)	SDS (5%)
Pronase (125 μg/ml)	*n*-Lauroylsarcosine (5%)
Papain (100 μg/ml)[b]	Triton X-100 (5%)
Subtilisin, pH 9.0 (1,000 μg/ml)	
Trypsin (1,000 μg/ml)	
Chondroitinase (250 μg/ml)	Other agents
Hyaluronidase (250 μg/ml)	Urea (6 M)
N-acetylhexosaminidase (200 μg/ml)	NaCl (2 M)
Xanthine oxidase or hypoxanthine (100 μg/ml)	EDTA (50 mM)
	Guanidine thiocyanate (3 M)
Solvents	Guanidine hydrochloride (6 M)
Hot trichloroacetic acid (5%)	$NaBH_4$ (0.3 M)
Hot phenol (45%)	
Chloroform-methanol (1:2)	
Boiling H_2O	

[a] Suspensions of the sheath of *L. discophora* were treated with the agents as described in Materials and Methods.
[b] Treatment with papain caused a 20% decrease in OD_{600} in 30 min at 25°C, but the sheaths remained unchanged in appearance under phase contrast microscopy.

as shown previously (15), but the sheath structure remained intact with little change in phase density as observed microscopically. The detergents listed in Table 1 had all been used in developing the sheath isolation protocol (15); therefore, it was not surprising that none of them had a substantial effect on the sheath structure after isolation, even at concentrations five times higher than those used for isolation. Urea (6 M) caused a small, 10%, drop in OD_{600} but no perceptible changes in phase density. Likewise, 3 M guanidine thiocyanate and 6 M guanidine-HCl also had no measurable effects. EDTA was used at a concentration of 2.5 mM to isolate sheaths. The 50 mM concentration reported in Table 1 caused no change in OD_{600}. Treatment with 0.3 M $NaBH_4$ caused the sheaths in suspension to coagulate but did not alter their phase density in the light microscope.

The sheaths were susceptible to hydrolysis with 1 N NaOH at 50°C (80% decrease in OD_{600} in less than 10 min); no sheath structures were observed by phase contrast microscopy. Hydrolysis with 1 N HCl at 50°C was less effective, causing a 40% decrease in OD_{600} after 1 h.

Effect of other reagents. A variety of enzymes, solvents, detergents, and other reagents were tested to determine their effect on sheath structure as revealed by changes in the appearance of the sheath in phase contrast light microscopy or a decrease in OD_{600}. Those that had little or no significant effect on absorbance or phase density are listed in Table 1. Among the enzymes tested, only papain (100 $\mu g/ml^{-1}$) caused a 20% decrease in the OD_{600} of the sheath suspensions in 30 min but no change in phase density. None of the other proteases tested caused any noticeable change in OD_{600} or in phase density. Treatment with lysozyme, chondroitinase, hyaluronidase, and *N*-acetylhexosaminidase also resulted in no detectable optical-density changes or release of chondroitin, hyaluronic acid, or *N*-acetylhexosamines into the supernatant as indicated by chemical analysis for these compounds (15). Attempts to depolymerize the sheath with superoxide radicals generated with the hypoxanthine or xanthine oxidase system also had no observable effects.

The extent of dissociation at pH 9 was dependent upon DTT concentration. Incubation in 20 mM bicine buffer at pH 9 alone with no DTT present caused a 9% decrease in OD_{600} after 30 min at 40°C and 30% recovery of protein in the supernatant after centrifugation at 8,800 × *g* for 4 min to remove intact sheaths. Incubation with 0.5 mM DTT caused a 16% decrease in OD_{600} and a 54% release of the protein into the supernatant. Incubation of the sheath in 5 mM DTT caused a 70% decrease in OD_{600} within 5 min (Fig. 1); 83% of the protein was released. After treatment with concentrations of DTT greater than 1 mM at pH 9.0, little or no sheath material was found in the pellet after centrifugation at 20,000 × *g* for 20 min.

Other disulfide bond-reducing agents also caused the sheath to dissociate. β-Mercaptoethanol behaved similarly to DTT, causing an approximately 60% decrease in OD_{600} within 15 min at pH 9.0 and 40°C. At pH 7.0, β-mercaptoethanol also caused a decrease in OD_{600}, but the sheath remained intact as determined by phase contrast microscopy. One hour of treatment with 20 mM Na_2SO_3 or 10 mM KCN at pH 9.0 and 50°C caused 25 and 20% decreases in OD_{600}, respectively. The dissociation rates were much slower than those for DTT or β-mercaptoethanol. After 24 h of treatment with these reagents, very little intact sheath was observed. This is in keeping with cyanide and sulfite being weaker disulfide bond-reducing agents than DTT or β-mercaptoethanol (22, 41).

Ultrastructure of DTT-treated sheaths. The effects of DTT on dissociation of the sheath fabric were clearly seen by TEM of metal-shadowed material. Controls treated only with pH 9 buffer resulted in essentially no change in the appearance of sheaths (compare Fig. 2A with Fig. 3B in reference 15). In samples treated with 5.0 mM DTT, a few partly dissociated sheaths were seen (Fig. 2B); however, the majority of material on the EM grids consisted of unorganized masses of fibrils. Individual, metal-coated fibrils measured 6.5 nm (standard deviation = 2.1; n = 19) in diameter, which is consistent with measurements of fibrils in intact sheaths (15). The fibrils were of indeterminate length. Most appeared to be very long. Individual fibrils could be measured for 5 μm or more without an end being found (Fig. 2B and C). Observations of numerous metal-shadowed grids showed that the extent of dissociation of individual sheaths depended on DTT concentration. When samples were treated with 0.5 or 1.0 mM DTT for 30 min, intact, or nearly intact, sheaths were plentiful on EM grids; however, at concentrations of 2.0 mM DTT or greater, intact sheaths were never observed and partially intact sheaths such as those shown in Fig. 2B were only rarely seen. Instead, the grids were uniformly covered with sheath fibrils (Fig. 2C).

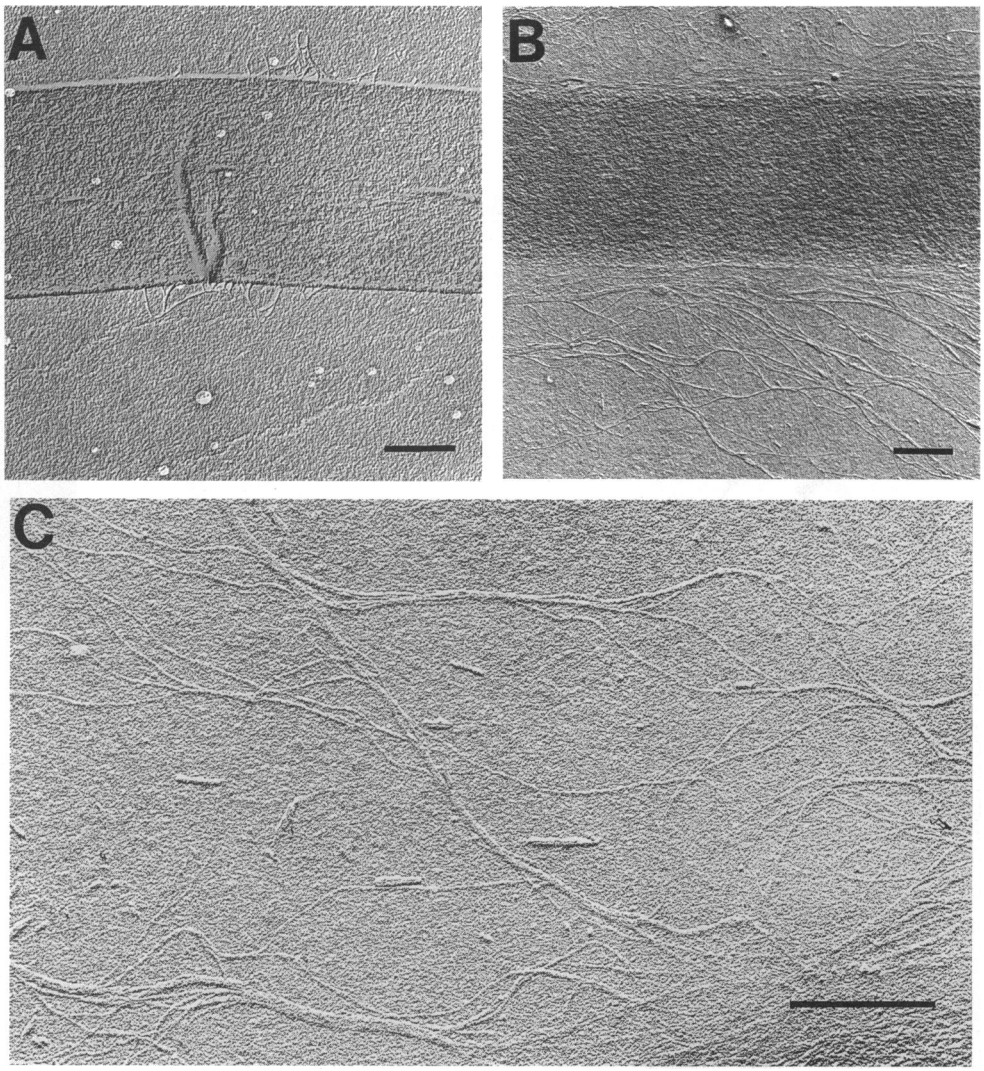

FIG. 2. Dissociation fibrils of *L. discophora* SP-6 sheaths as visualized by TEM of metal-shadowed samples. After treatment of a sheath suspension as indicated, an EM grid was floated on a drop of suspension for 2 min. The grid was removed, air dried, and shadowed with platinum-palladium (80:20) in a vacuum evaporator. (A) Untreated sheath incubated without DTT but with pH 9.0 buffer for 30 min at 40°C. Bar = 0.5 μm. (B and C) For sheath incubated with 5 mM DTT and pH 9.0 buffer for 30 min at 40°C, very few partially dissociated sheaths (B) and abundant individual fibrils (C) were seen. Bar = 0.5 μm.

Quantitation of SH and disulfide. Assaying the number of disulfides in isolated sheaths was complicated by the polymeric and anionic nature of the sheath matrix. Measurement of the increase of A_{310} during the oxidation of DTT (21) proved to be the most successful method (Table 2). However, filtration was necessary to remove the sheath fibrils produced during DTT treatment since they elevated the background light scattering to levels that exceeded the change in A_{310} from oxidation of DTT. Presence of fibrils in the filtrate was easily checked by acidifying it with 1 N HCl, which caused an immediate, visible aggregation of the fibrils, if they were present. As expected, alkylation of free SH groups with iodoacetic acid reduced the concentration of free SH groups detected by DTNB, but it had little effect on the concentration of disulfides detected by the DTT assay (Table 2). Approximately 20% of the total SH in the sheath occurred as free SH groups as determined by DTNB (Table 2).

Further evidence for the presence of free SH groups in the

TABLE 2. Estimation of free SH and disulfide and total SH content in the sheath material of *L. discophora* SP-6

Reagent[a]	μmol of SH[b] or S-S[c] mg of protein^{-1}[d]	
	Untreated sheaths [*n*]	Alkylated sheaths [*n*]
DTNB	0.8 (0.05) [5]	0.06 (0.02) [2]
DTT	2.2 (0.73) [3]	2.4 [1]
Total SH [2(S-S) + SH]	5.2	

[a] DTNB was used to detect SH, and DTT was used to detect disulfide (S-S).
[b] Determined by the method of Jocelyn (22).
[c] S-S, disulfide. Determined by measuring increase in A_{310} of filtered suspension after treatment with DTT as described in Materials and Methods.
[d] Numbers in parentheses are standard deviations.

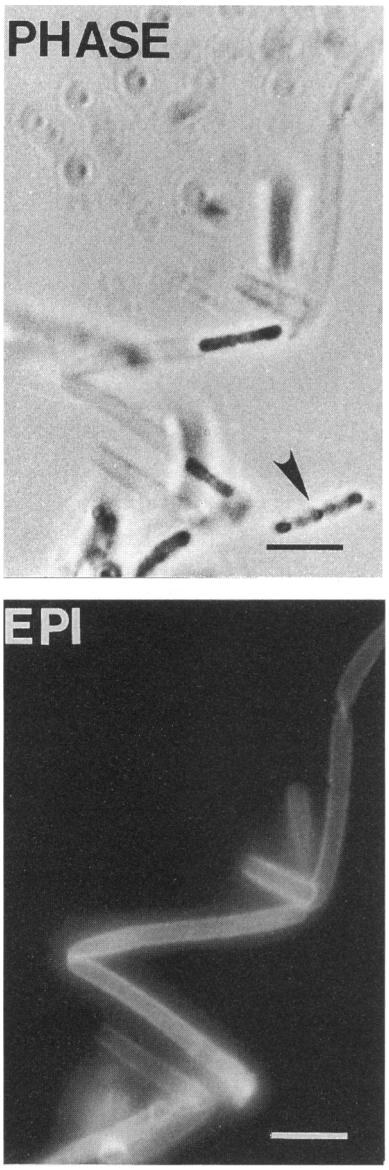

FIG. 3. Fluorescent labeling of SH groups in *L. discophora* SP-6 sheaths with the SH-specific probe, FM. Phase contrast (PHASE) (upper panel) and epifluorescence (EPI) (lower panel) images of the same microscope field showed that the sheath was intensely labeled while free cells (arrow, upper panel) were not. Bars = 5 μm.

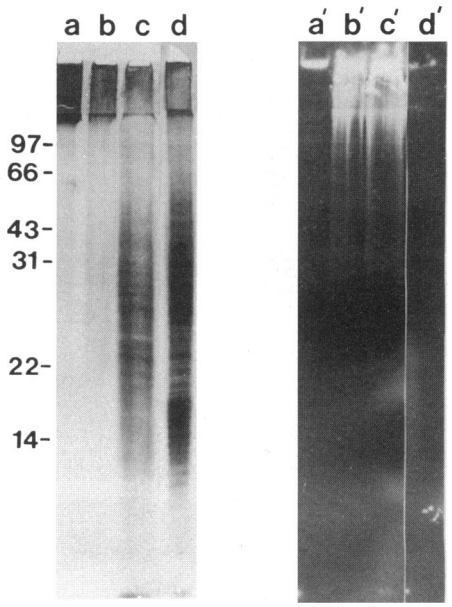

FIG. 4. SDS-PAGE electrophoretogram of [^{14}C]iodoacetamide-labeled samples of *L. discophora* SP-6 sheaths. On the left is a silver-stained gel, and on the right is an autoradiogram of the same gel. Each lane was loaded with approximately 10 μg of total protein. Lanes a and a′ contain whole sheaths and buffer without DTT; lanes b and b′ contain whole sheaths and treatment buffer with 5 mM DTT; lanes c and c′ contain sheaths dissociated with 5 mM DTT before being loaded on the gel; lanes d and d′ contain the same DTT-dissociated sheaths pretreated with [^{12}C]iodoacetamide before being labeled with [^{14}C]iodoacetamide. MW is indicated on the left.

sheath was provided by the SH-specific FM probe. Treatment with the FM reagent at 25 molar excess relative to the SH concentration caused sheaths to fluoresce bright apple green, typical of fluorescein (Fig. 3). Swarmer cells outside sheaths and cells within the sheaths fluoresced weakly. Alkylation of free SH groups with iodoacetic acid before treatment with the FM reagent strongly diminished the fluorescence of FM-treated sheaths (not illustrated).

SDS-PAGE analysis of [^{14}C]iodoacetamide autoradiography and PAS staining. Silver staining of whole and dissociated sheaths after SDS-PAGE revealed that very little material from undissociated whole-sheath suspensions entered the resolving gel, regardless of whether DTT was present in the treatment buffer (compare lanes a and b in Fig. 4). After sheaths were subjected to dissociation and alkylation (Fig. 4, lanes c and d), a large number of bands appeared within a smear of silver-stained material in the middle and lower parts of the resolving gel. These bands were commonly found to be concentrated in a zone corresponding to the molecular-mass range of 14 to 45 kDa.

The autoradiogram of the same material labeled with [^{14}C]iodoacetamide showed that when DTT was not present in the treatment buffer, very little of the ^{14}C label entered the stacking gel from whole sheaths (Fig. 4, lane a′), while the aggregated material in the loading well autoradiographed strongly, indicating that labeling had indeed occurred. When 10 mM DTT was added to treatment buffer containing labeled whole sheaths (Fig. 4, lane b′), some ^{14}C-labeled material did enter the stacking gel and faint ^{14}C-containing bands appeared in a smear above 43 kDa in the resolving gel. Dissociation of the sheath with DTT before labeling with [^{14}C]iodoacetamide (Fig. 4, lane c′) showed almost exactly the same effect as dissociation after labeling (Fig. 4, lane b′), suggesting that the same SH groups were labeled before and after reduction of disulfide bonds. The ^{14}C-labeling pattern of the autoradiograms was very different from the zones of silver-stained material (compare lanes b and b′ and lanes c and c′ in Fig. 4), indicating that the low-MW bands seen in the silver-stained gels did not contain SH groups accessible to the labeling reagent, despite the release of this material by the SH-producing DTT dissociation process. The negative control, which was prelabeled with [^{12}C]iodoacetamide before treatment with the ^{14}C-labeled reagent, showed very little labeling in the autoradiograms (Fig. 4, lane d′), indicating that the ^{14}C labeling was blocked by this SH-specific reagent.

Pronase treatment of dissociated, alkylated sheath fibrils slightly altered migration on gels, as indicated by their silver stain banding patterns (compare Fig. 4 and 5). Numerous bands still appeared in the 14- to 45-kDa range; however, bands above 45 kDa were diminished while bands in the 14- to 30-kDa region were intensified.

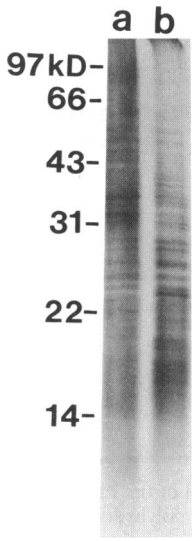

FIG. 5. SDS-PAGE of DTT-dissociated iodoacetic acid-alkylated sheaths before (lane a) and after (lane b) incubation with 50 μg of pronase per ml for 5 h at 37°C. Both lanes were loaded with approximately 10 μg of total protein. The silver stain banding pattern was altered slightly after treatment (lane b).

DISCUSSION

The sheath of *L. discophora* SP-6 is a very stable biological structure. It was found to resist degradation by a variety of lytic enzymes and other denaturing reagents (Table 1). The sheath was, however, very susceptible to degradation by disulfide bond-reducing agents. Our results suggest that disulfide bonds are responsible for cross-linking the sheath structural fibrils to form a cohesive fabric that maintains the stability of the sheath structure. Given that disulfide bonds are important in maintaining integrity in other bacterial structures, such as the spore coat of *Bacillus* spp. (3), the elementary bodies in *Chlamydia* spp. (28), and the proteinaceous hoop subunits of the sheath of the archaeon *Methanospirillum hungatei* GP1 (36, 37), and that among eukaryotes a number of disulfide-bonded structural heteropolymers, including keratin (39) and mucin-like glycoproteins (7), are known, it is not surprising to find that a robust structure such as the sheath of *L. discophora* SP-6 is maintained by disulfide bonds.

Dissociation of the sheath fabric as observed in metal-shadowed samples by TEM showed that individual fibrils remained intact after sheath dissociation was complete. In addition, our analyses with the SH reagents DTT and DTNB (Table 2) and the specific fluorescent labeling of the sheath with FM (Fig. 3) demonstrated that not all of the SH groups in the sheath were involved in disulfide bonding. At least 20% of them may exist as free SH groups. These results suggest that sheath assembly may take place by disulfide cross-linking after the cell excretes individual sheath fibrils into the growing sheath matrix. According to this hypothesis, the sheath fibrils would be "glued" together via disulfide bond formation in the more oxidizing regions outside the cell cytoplasm after they are excreted and possibly processed through the periplasm of the gram-negative cell wall. Indeed, the cytoplasm of bacteria is considered to be too reducing an environment for disulfide synthesis from SH groups (16). It may be further speculated that the synthesis of disulfide bonds during sheath assembly could involve the participation of an extracellular protein disulfide oxidoreductase or a protein disulfide isomerase (2, 4, 29, 40).

The results of electrophoretic studies of silver-stained intact and dissociated sheaths suggest that the dissociation procedure releases material of relatively low MW. Also, the labeling studies with [^{14}C]iodoacetamide showed that this low-MW material contained few, if any, labeled SH groups. Most of the labeled SH-containing material did not enter the gel, suggesting it was bound in large aggregates. DTT treatment did have some effect on dissociating the aggregated ^{14}C-containing material, but the bulk of labeled material still did not enter the resolving gel after disaggregation (Fig. 4). This evidence, plus our previous evidence showing the presence of only a few Coomassie blue-staining protein bands in SDS-PAGE of whole sheaths (see Fig. 6 in reference 15), suggests that the low-molecular-mass material that migrated to the 14- to 45-kDa range in the silver-stained gels obtained in this study contained relatively small amounts of protein. We speculate that these bands contained mostly heteropolysaccharides, which are known to be stained by the silver stain reagents (26). This idea is supported by the fact that pronase had only a slight effect on the migration patterns of the silver-staining material (Fig. 5). It is possible that the low-MW material was removed from the sheath by the DTT treatment. Since this material did not contain significant amounts of SH, it may, in fact, contain capsular polymers (15) rather than the sheath fibrils. Clarification of this point awaits further analysis of the structural chemistry of the sheath.

It was surprising that the dissociated sheath material was not stained with the PAS staining reagents while the positive control (horseradish peroxidase) behaved as expected. The PAS stain is based on periodic acid oxidation of vicinal diols to aldehydes on sugar moieties in a polysaccharide (18). The aldehydes then react with a Schiff reagent to produce the magenta-colored dye product. One possible explanation for the lack of PAS staining of the sheath fibril polysaccharides is that the principal sugar moieties, uronic acids and amino sugars (15), are substituted such that the vicinal diols necessary for PAS staining are not available for oxidation.

Additional work on purified sheath fibrils (12) showed that they contained approximately the same ratios of protein (20 to 25%), amino sugar (15 to 17%), and uronic acid (20%) on a dry-weight basis as did the isolated whole sheaths (15). The purified fibrils also were found to be more highly enriched in cysteine than the intact sheaths (12). They also contained high-MW material that stained with alcian blue on SDS-PAGE gels. These results support the idea that the fibrils are composed principally of anionic glycosaminoglycan or proteoglycan components (26).

From the results presented in this and our previous work (12, 14, 15), we propose a working model to summarize our current knowledge of the structure of the *L. discophora* SP-6 sheath (Fig. 6). In this model, the matrix of the sheath consists of two layers, an inner layer of tightly woven structural fibrils and an outer layer of diffuse capsular material. Whether the outer layer is actually a diffuse array of the same fibrils that make up the inner layer or a chemically unique capsule is unknown. The results of chemical analysis (15) showed that uronic acids are abundant in the whole sheaths, and the alcian

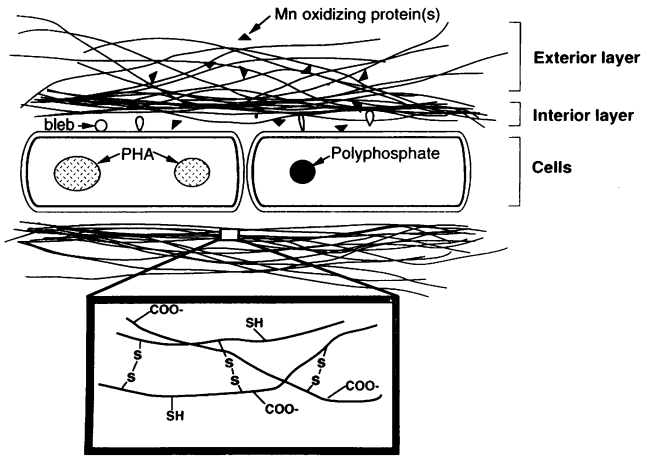

FIG. 6. Working model of the molecular structure of the sheath of *L. discophora* SP-6. PHA, polyhydroxyalkanoate.

blue staining results described above suggest that the fibrils contain anionic polymers. These results further suggest that there are numerous free carboxylate groups in the sheath fabric that account for its anionic character. Interfibril disulfide bonds appear to be the covalent glue holding the structural fibrils together in the inner layer. In addition, it appears there is a significant concentration of free SH groups distributed throughout the sheath. Whether free SH groups are located in the inner or outer layer or whether they are evenly distributed between the layers is unknown. Both the free SH groups and the free carboxyl groups provide numerous sites for binding of Mn^{2+} and Fe^{2+} as well as other cationic metal species. This model fits well with our previous findings (14) that the sheath is the primary site for Mn oxidation by *L. discophora* SP-6 but that the excreted Mn-oxidizing protein is not integrated covalently in the sheath fabric. We now propose that the anionic fibrils of the sheath bind Mn^{2+} in such a manner that the Mn-oxidizing protein can catalyze its oxidation to Mn oxide. A similar model may apply to Fe^{2+} binding and oxidation in the sheath by the recently discovered extracellular iron-oxidizing protein of *L. discophora* SS-1 (8). Thus, the combined chemical and structural properties of the sheath help to explain how this multifunctional and resilient bacterial structure can serve simultaneously as a site for extracellular Mn and Fe oxidation as well as a "cocoon" to protect the *L. discophora* cells from predation, toxic oxygen species, and other environmental stresses.

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